Kidney International, Vol. 68 (2005), pp. 1137–1147

ION CHANNELS – MEMBRANE TRANSPORT – INTEGRATIVE PHYSIOLOGY

Role of membrane microdomains in PTH-mediated down-regulation of NaPi-IIa in opossum kidney cells

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Background. Parathyroid hormone (PTH) rapidly down-regulates type IIa sodium-dependent phosphate transporter (NaPi-IIa) via an endocytic pathway. Since the relationship between PTH signaling and NaPi-IIa endocytosis has not been explored, we investigated the role of membrane microdomains in this process.

Methods. We examined the submembrane localization of NaPi-IIa in opossum kidney (OK-N2) cells that stably expressed human NaPi-IIa, and searched for a PTH-induced specific phosphorylating substrate on their membrane microdomains by immunoblotting with specific antibody against phospho substrates of protein kinases.

Results. We found that NaPi-IIa was primarily localized in low-density membrane (LDM) domains of the plasma membrane; PTH reduced the levels of immunoreactive NaPi-IIa in these domains. Furthermore, PTH activated both protein kinase A (PKA) and protein kinase Cα (PKCa) and increased the phosphorylation of 250 kD and 80 kD substrates; this latter substrate was identified as ezrin, which a member of the ezrin-radixin-moesin (ERM) protein family. In response to PTH, ezrin was phosphorylated by both PKA and PKC. Dominant negative ezrin blocked the reduction in NaPi-IIa expression in the LDM domains that was induced by PTH.

Conclusion. These data suggest that NaPi-IIa and PTH-induced phosphorylated proteins that include ezrin are compartmentalized in LDM microdomains. This compartmentalization may play an important role in the down-regulation of NaPi-IIa via endocytosis.

Serum inorganic phosphate (Pi) homeostasis is maintained by Pi reabsorption that is regulated by a sodium-dependent Pi transport system in the kidney [1, 2]. Type IIa sodium-dependent Pi transporter (NaPi-IIa) mediates most of the Pi reabsorption in the kidney and is tightly regulated by various Pi-regulating hormones and dietary phosphate intake [1–3].

Parathyroid hormone (PTH) is an important regulator of Pi reabsorption in renal proximal tubules [1–3] and was shown to rapidly reduce their NaPi-IIa activity by stimulating apical endocytosis [4]. Down-regulation of NaPi-IIa was reported to be mediated by protein kinase A (PKA) and/or protein kinase C (PKC) activation in these cells [1, 2, 5]. However, the exact mechanism by which PTH mediates NaPi-IIa endocytosis is still unclear.

It has been suggested that membrane microdomains such as lipid rafts and caveolae play a role in the down-regulation of NaPi-IIa, since changes in the lipid composition (cholesterol/glycosphingolipids) of brush border membranes was shown to modulate NaPi-IIa transport activity [6]. Lipid rafts and caveolae are membrane microdomains that are enriched for cholesterol and sphingolipids [7, 8] and are characterized as low-density membrane (LDM) or Triton X-100 insoluble membrane domains. Compartmentalization of certain signal transduction molecules into these microdomains was shown to play a role in the regulation of microdomain-mediated membrane trafficking [7, 8]. As an example of this, the presence of PKA and PKCα in caveolae was shown to be important for the regulation of caveolae-dependent endocytosis [9–11].

We hypothesized that in addition to NaPi-IIa, the PTH receptor and its signal transducing molecules, including PKA and PKC, as well as their substrates might similarly be localized in membrane microdomains where they play a role in the endocytosis-mediated down-regulation of NaPi-IIa. Our data showed that NaPi-IIa and PTH-mediated signal transduction molecules are localized in LDM domains, and that PTH can increase the PKA- and PKC-mediated phosphorylation of specific substrates in these domains that mediate the down-regulation of NaPi-IIa.

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Key words: sodium-dependent phosphate transporter, parathyroid hormone, renal proximal tubular cells, endocytosis, membrane microdomains.

Received for publication November 16, 2004
and in revised form March 7, 2005
Accepted for publication April 14, 2005

METHODS

Antibodies

The anti-NaPi-2 polyclonal antibody that we used in this study was one that was previously generated in our laboratory [13]. Anti-FLAG monoclonal antibody was purchased from Sigma Aldrich Japan (Tokyo, Japan). Anti-myc-tag polyclonal antibody was purchased from Upstate Biotechnology Inc (Lake Placid, NY, USA) while the anticaveolin polyclonal antibody, antitransferrin receptor monoclonal antibody, anti-PKA (α catalytic subunit) monoclonal antibodies, and anti-PKCα monoclonal antibodies were purchased from BD Pharmingen (San Diego, CA, USA). The antiphospho-(serine) PKC substrate polyclonal antibody and anti-phospho-(serine/threonine) PKA substrate polyclonal antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antiopossum PTH receptor monoclonal antibody [opossum kidney-1 (OK-1)] was purchased from Covance (Berkeley, CA, USA). Antiezrin antibody was purchased from Zymed (South San Francisco, CA, USA), and antiphospho ezrin (Thr567) antibody from Chemicon (Temecula, CA, USA). For the Western blot analyses, horseradish peroxidase–conjugated antimouse or antirabbit IgG (Bio-Rad Japan, Tokyo, Japan) was used as the secondary antibody. Alexa555-conjugated goat antimouse IgG (Molecular Probe, Eugene, OR, USA) was used for immunofluorescence analysis.

Plasmid construction

Human NaPi-IIa (NaPi-3) cDNA [14] was inserted into the polycytomegalovirus (pCMV)-Tag2 vector (Stratagene, La Jolla, CA, USA) at the *Bgl*II-*Xho*I site. A cDNA fragment containing the human NaPi-IIa gene and the eptitope tag (FLAG tag) was excised by restriction endonucleases *Hind*II, and inserted into the pIRES-neo2 vector (Clonetech, Palo Alto, CA, USA) at the *Eco* RV site (hereafter, pIRES-NaPi-IIa-FLAG).

Cell culture, transfection, and establishment of stable cell lines

OK-P cells were kindly provided by Dr. Amemiya (Jichi Medical School, Tochigi, Japan). The antirat NaPi-IIa antibody (anti-NaPi-2 polyclonal antibody) developed in our previous study [13] reacts with human and rat NaPi-IIa but not with endogenous opossum NaPi-IIa in OK-P cells. The cells were maintained in plastic culture dishes in Dulbecco's modified Eagle's medium (DMEM) (LifeTechnologies, Gaithersburg, MD, USA) containing 25 mmol/L Hepes, 4000 mg/mL glucose, 1 mmol/L sodium pyruvate, 50 μg/mL penicillin, 50 IU/mL streptomycin, and 10% (vol/vol) fetal bovine serum (FBS) (Sigma-Aldrich Japan, Tokyo, Japan) at 37°C in a humidified atmosphere of 5% CO_2. Transfection was carried out using Lipofectamine-plus (LifeTechnologies). To select stable cell lines expressing human NaPi-IIa with FLAG tag, pIRES-NaPi-IIa-FLAG was transfected into OK-P cells, after which they were cultured in the presence of 2000 μg/mL of G418 for 10 days [15]. Following G418 selection, the cells were subjected to subcloning by limited dilution. Four cell lines (OK-N1 to OK-N4) were generated, and the response of each to PTH was characterized. The OK-N2 cell line expressed a moderate amount of human NaPi-IIa and it was this cell line that was used in our experiments. Cells were used within eight passages of their establishment as a cell line.

Treatment with PTH and other chemicals

OK-N2 cells were seeded into 100 mm culture dishes in the above media (maintenance media) that was supplemented with 2000 μg/mL of G418, and were grown to subconfluency. The cells were preincubated in working media [maintenance medium containing 0.1% bovine serum albumin (BSA) instead of FBS] for 12 hours, after which they were treated with 10^{-7} mol/L PTH (1-34 fragment) (kindly provided from Asahi Kasei Pharma, Tokyo, Japan) at 37°C for either 0, 15, 30, or 60 minutes. In some cultures, chlorpromazine (1 μmol/L), an inhibitor of clathrin-dependent endocytosis, 3-methyl-β-cyclodextrin (0.5 mmol/L), an inhibitor of LDM domains that acts by chelating cholesterol, cytochalasin D (0.5 μmol/L), a facilitator of actin depolymerization, or nocodazole (1 μmol/L), a facilitator of microtubular depolymerization was added for 5 minutes before the cells were treated with PTH for 30 minutes. Gö6976 (10 μmol/L) (Calbiochem, San Diego, CA, USA), a specific inhibitor of PKC, or H-89 (100 μmol/L) (Calbiochem), a specific inhibitor of PKA was added to other cultures for 5 minutes before cells were treated with PTH for 20 minutes.

Pi uptake

Pi uptake was measured in OK-N2 cells grown to confluency in 24-well plastic plates as previously described [15]. Transport rates were expressed as pmol Pi per 10^4 cells per minute.

Cell fractionation

The detergent-free caveolae isolation method was used for cell fractionation [16], with slight modifications as described previously [9]. All experiments were carried out at 4°C. Cells were homogenized in ice-cold buffer A [0.25 mol/L sucrose, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), and 20 mmol/L tricine, pH 7.8] with a dounce homogenizer. The homogenate was then centrifuged at 1000*g* for 10 minutes and the supernatant extracted and designated as the postnuclear supernatant

fraction (PNS). This fraction was subjected to gradient centrifugation in a 30% percoll solution, prepared in buffer A, at 84,000*g* for 30 minutes to obtain plasma membranes. Sonicated plasma membranes were subjected to a 10% to 20% OptiPrep (LifeTechnologies) gradient centrifugation (OptiPrep 1) at 52,000*g* for 90 minutes, after which the fractions were either sequentially (1 mL/fraction) or collectively (the top 5 mL comprising gradient fractions 1 to 5) harvested. The former were subjected to Western blot analysis and protein determination while the latter were subjected to 5%/30% OptiPrep gradient centrifugation (hereafter referred to as OptiPrep 2). The opaque band located just above the 5% interface was collected and designated as the LDM domains. Pooled fractions 6 to 10 from OptiPrep 1 were designated as the high-density membrane (HDM) domains.

Electrophoresis and Western blots

Samples were separated using 7% to 15% linear-gradient sodium dodecyl sulfate (SDS)-polyacrylamide gels and were transferred to a polyvinlydine difluoride (PVDF) membrane (Immobilon-P) (Millipore Japan, Tokyo, Japan) which was then incubated with primary antibodies for 1h at room temperature. After washing out the unbound antibodies, the membrane was incubated with the appropriate horseradish peroxidase–conjugated antimouse or antirabbit IgG for 1 hour at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL) (Amersham-Pharmacia Biotech Japan, Tokyo, Japan) and were analyzed with an LAS-1000 lumino-image analyzer (Fujifilm, Tokyo, Japan); densitometric analysis was also carried out.

Immunocytochemical analysis

OK-N2 cells that were grown on plastic coverslips were incubated with 100 μg/mL of Alexa488-labeled cholera toxin B subunit (CTB) (Molecular Probe) at 4°C for 30 minutes before fixation, after which the cells were fixed for 30 minutes with 3% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS). The cells were then permeabilized with 0.1% Triton X-100 in PBS for 10 minutes on ice. After blocking with 0.8% BSA in PBS for 1 hour, the cells were incubated with anti-FLAG tag monoclonal antibody for 1 hour after which they were washed and labeled with Alexa555-labeled antimouse IgG. The cells were then washed and mounted on glass slides with Aqua/poly mount (PolyScience, Niles, IL, USA). Slides were analyzed and photographed using a Leica Confocal Microscope (TCS-SL) (Leica Microsystems GmbH, Mannheim, Germany) in the sequential mode. The x and y plane images (512 × 512 pixels per image) were obtained in the x, y, and z scan mode, and 18 z section images were taken. The top 1 to 4 z section images or bottom 14 to 18 z section images were projected onto one x and y plane image from top to bottom. X and z plane and y and z plane images were obtained from stacked 18 z section images. Foci where CTB or NaPi-IIa was stained and where their staining overlapped were counted in 12 images from four independent experiments. The coincidence ratio was calculated using following equation: ratio (%) = (the number of coincident foci)/(the number of NaPi-IIa foci) × 100.

Determination of protein concentration

Protein concentrations were determined using the Bio-Rad Bradford Assay (Bio-Rad Japan, Tokyo, Japan) with a BSA standard.

Cyclic adenosine monophosphate (cAMP) assay and cholesterol uptake assay

cAMP concentration was determined using a cAMP Assay Kit (Amersham Pharmacia Biotech). Cholesterol uptake was assessed as described previously [17] using DiI-low-density lipoprotein (LDL) (Biomedical Technologies, Stoughton, MA, USA).

Amino acid sequence analysis

Amino acid sequence analysis was carried out by the APRO Science Company (Tokushima, Japan). The 80 kD band that was separated by SDS-polyacrylamide gel electrophoresis (PAGE) was subjected to "in gel" digestion with lysylendopeptidase and the resulting peptides were separated by reverse-phase high-performance liquid chromatography (HPLC) [18]. Amino acid sequence analysis of the peptides was carried out using a Procise 494cLC protein sequencer (Applied Biosystems Japan, Tokyo, Japan).

Immunoprecipitation of ezrin

Ezrin was immunoprecipitated from LDM domains that were obtained from Opti-Prep 2 cells that were incubated in the presence or absence of 10^{-7} mol/L PTH for 20 minutes. LDM domains were precipitated by ultracentrifugation at 300,000 × *g* for 1 hour and solubilized with TETN150/OG (25 mmol/L Tris-HCl, pH 7.5, 5 mmol/L EDTA, 150 mmol/L NaCl, 1% Triton X-100, and 60 mmol/L n-octyl-β-D-glucopyranoside) on ice. The solubilized samples were incubated with antiezrin monoclonal antibodies at 4°C overnight, after which the immunocomplexes were precipitated using protein A sepharose CL-4B that was precoated with BSA. After washing out the unbound proteins 4 times with TETN250 (25 mmol/L Tris-HCl, pH 7.5, 5 mmol/L EDTA, 250 mmol/L NaCl, and 1% Triton X-100), the precipitates were suspended in 2 × SDS sample buffer and subjected to Western blotting using antiezrin monoclonal

antibody, antiphospho (serine/threonine) PKA substrate polyclonal antibody, or antiphospho (serine) PKC substrate polyclonal antibody, as described above.

Cloning and transient expression of N-terminal half of ezrin (dominant-negative ezrin)

The N-terminal half of ezrin cDNA was generated from the human colon cancer cell line, Caco-2 by reverse transcription-polymerase chain reaction (RT-PCR) using the following primers that contained their restriction enzyme cleavage site either at *Xba*I for sense or *Hind*III for antisense-sense 5′-GCTCTAGACCGAAAATG CCGAAACCAATC-3′ (corresponding to −6 to +15, A for translation initiation site is +1), antisense 5′-CCCAAGCTTCTCCCGGGCCTGG GCCTTCAT-3′ (+913 to +933). Amplified cDNA fragments were digested with *Xba*I and *Hind*III and subcloned into the pcDNA 3.1 (−) myc-His A vector (Invitrogen), which was transiently transfected into OK-N2 cells with Lipofectamine-plus. Transfection efficiency was estimated to be more than 60% using a green fluorescence protein (GFP) expression vector. Two days after transfection, the cells were treated with 10^{-7} mol/L PTH for 30 minutes after which they were subjected to LDM domain preparation. The myc-tagged dominant-negative ezrin was detected by anti-myc-tag polyclonal antibodies.

Statistical analysis

Data are presented as the means ± SEM. Statistical significance ($P < 0.05$) between groups was determined by analysis of variance (ANOVA) followed by post hoc testing using Fisher's protected least significant difference (PLSD) procedure for multiple comparisons.

RESULTS

We confirmed that Pi transport in OK-N2 cells was approximately 2.5-fold greater than that seen in parental OK-P cells (4.93 ± 0.17 vs. 1.91 ± 0.29 pmol/min/10^4 cells, respectively), and that treatment with 10^{-7} mol/L PTH for 30 minutes reduced Pi transport activity in OK-N2 cells by about 50% of untreated OK-N2 cells (2.63 ± 0.087 pmol/min/10^4 cells) ($P < 0.01$). These data validated the use of this cell line for the study of PTH regulation of NaPi-IIa.

Biochemical and immunocytochemical analysis of the localization of NaPi-IIa

To determine the location of NaPi-IIa in OK-N2 cells, purified plasma membranes were subjected to OptiPrep gradient centrifugation (OptiPrep 1). Western blots of each fraction (total protein) from the OptiPrep 1 gradients showed that most of the NaPi-IIa was localized in the LDM fractions as same as caveolin (Fig. 1A) (NaPi-IIa, lanes 1 to 5). On the other hand, transferrin receptor that is located in clathrin-coated pits was detected in the heavier fractions (lanes 6 to 10).

We also examined the location of NaPi-IIa in OK-N2 cells by immunofluorescence using the localization of CTB as a reference control. CTB, a well-characterized membrane microdomain marker protein that binds to GM_1 ganglioside, was strongly localized to the apical plasma membrane (Fig. 1C). FLAG-tagged NaPi-IIa was similarly localized to the apical plasma membrane (Fig. 1D), in a pattern that was similar to that of CTB (Fig. 1E). We found that 77.5 ± 5.23% of the NaPi-IIa foci colocalized with CTB. We also found that most of the caveolin was localized in the basolateral membrane and was not obviously colocalized with NaPi-IIa (Fig. 2).

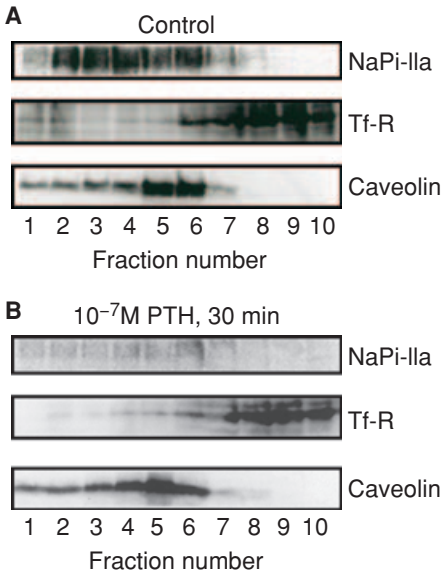
Effects of PTH on the localization of NaPi-IIa in LDM domains

We next examined whether PTH influenced the expression of NaPi-IIa in the low-density fractions in OptiPrep 1. Following treatment with 10^{-7} mol/L PTH for 30 minutes, the OptiPrep 1 fraction showed reduced levels of NaPi-IIa in the low-density fractions (Fig. 1B, lanes 1 to 5) compared to controls (Fig. 1A). PTH did not affect the localization of either the transferrin receptor or caveolin.

As shown in Figure 3A, a decrease in NaPi-IIa in plasma membranes and LDM domains in OptiPrep2 was observed in response to a 15-minute to 60-minute incubation with PTH. Densitometric analysis revealed that 50% of the NaPi-IIa disappeared from the LDM domains after 15 minutes, and 80% had decreased by 60 minutes; these results were statistically significant (Fig. 3B). Degradation of NaPi-IIa by PTH was not observed up through 60 minutes (PNS in Fig. 3), but did occur by 2 hours (data not shown).

Effects of 3-methyl-β-cyclodextrin, chlorpromazine, cytocharasin D, and nocodazole on the reduction in NaPi-IIa in the LDM domains by PTH

We examined the effects of the following four inhibitors on the down-regulation of NaPi-IIa: 3-methyl-β-cyclodextrin, chlorpromazine, cytochalasin D, and nocodazole. PTH significantly reduced the amount of NaPi-IIa in the LDM domains of OK-N2 cells (Fig. 4A and B). PTH also significantly inhibited Pi uptake activity (Fig. 4C). However, 3-methyl-β-cyclodextrin and cytochalasin D strongly inhibited PTH's influence on NaPi-IIa translocation (Fig. 4, lanes 3 and 5); 3-methyl-β-cyclodextrin did not affect PTH-mediated cAMP production (Fig. 5A). Chlorpromazine and nocodazole slightly inhibited the actions of PTH (Fig. 4, lanes 4 and 6). We confirmed that chlorpromazine inhibited DiI-LDL uptake in OK-N2 cells at the concentration used (1 μmol/L) (Fig. 5B). All four inhibitors had no effect on either the



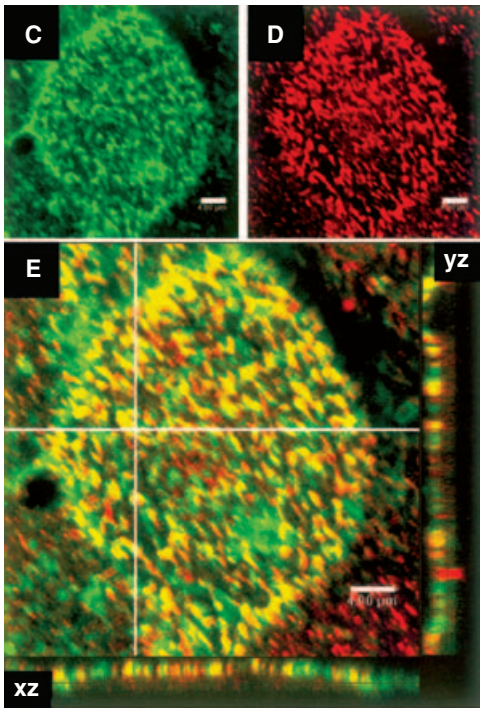


Fig. 1. Biochemical and immunocytochemical sub-membrane localization of sodium-dependent phosphate transporter (NaPi-IIa). (*A*) NaPi-IIa and caveolin in the low-density membrane (LDM) fractions derived from opossum kidney (OK-N2) cells were fractioned by OptiPrep 1 gradient centrifugation. Tf-R is antitransferrin receptor monoclonal antibody. (*B*) Effects of parathyroid hormone (PTH) on the localization of NaPi-IIa fractioned by OptiPrep 1 gradient centrifugation. OK-N2 cells were incubated in the presence of 10^{-7} mol/L parathyroid hormone (PTH) for 30 minutes. OK-N2 cells were immunocytochemically stained with cholera toxin B subunit (CTB) (*C*) or anti-FLAG-tag monoclonal antibody (*D*). (*E*) A merger of the images (C and D). Yellow dots indicate colocalization. Images were taken by confocal laser-scanning microscopy with a 63× oil immersion objective lens. The lower and right side panels show xy cross-sections (xz and yz planes) that were taken at the intersecting white lines (scale bar, 4 μm). The data presented are from four independent experiments.

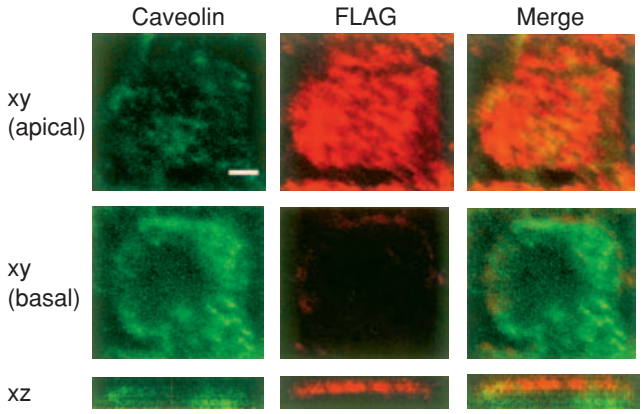


Fig. 2. Localization of caveolin and sodium-dependent phosphate transporter (NaPi-IIa) in opossum kidney (OK-N2) cells. OK-N2 cells were stained with anti-caveolin polyclonal antibody and anti-FLAG-tag monoclonal antibodies. Upper panels show the top xy sections, the middle panels show the bottom xy sections, and the bottom panels show the xz cross-sections. The data presented are from four independent experiments (scale bar, 4 μm).

amount of NaPi-IIa in the LDM domains or Pi uptake per se (Fig. 4, lanes 7 through 11).

Activation of PKCα and PKA in LDM domains by PTH

As shown in Figure 6A, the PTH receptor was localized in the LDM domains of OK-N2 cells. PTH and 8-bromo-cAMP reduced the amount of NaPi-IIa in the LDM domains (Fig. 6B and C) but increased the phosphorylation of PKA substrates at 80 kD and 250 kD in the LDM domains and at 175, 120, 80, and 65 kD in the PNS and cytosol, but not in the HDM domains (Fig. 6C). On the other hand, the amount of PKA in each fraction did not change as a result of PTH or 8-bromo-cAMP stimulation (Fig. 6B). Interestingly, PKCα levels increased in the LDM domains and decreased in the cytosol after PTH or phorbol 12,13-myristic acetate (PMA) treatment (Fig. 7A). The phosphorylation of PKC substrates was significantly increased at 250, 190, 110, and 80 kD in the LDM domains but not in the cytosol or HDM domains (Fig. 7B).

Amino acid sequence analysis and functional analysis of the 80 kD substrate

To identify the substrate at 80 kD, we performed amino acid sequence analysis on the 80 kD protein by "in-gel" digestion with lysylendopeptidase. We identified the peptide "IALLEEARRRKE" as a part of the candidate substrate. Homology search results showed that the peptide was identical to rat, mouse, and human ezrin. This compound fractionated into the LDM domains in a manner comparable to NaPi-IIa and caveolin (Fig. 8A), though PTH did not affect the localization of ezrin as it did NaPi-IIa (Fig. 8B).

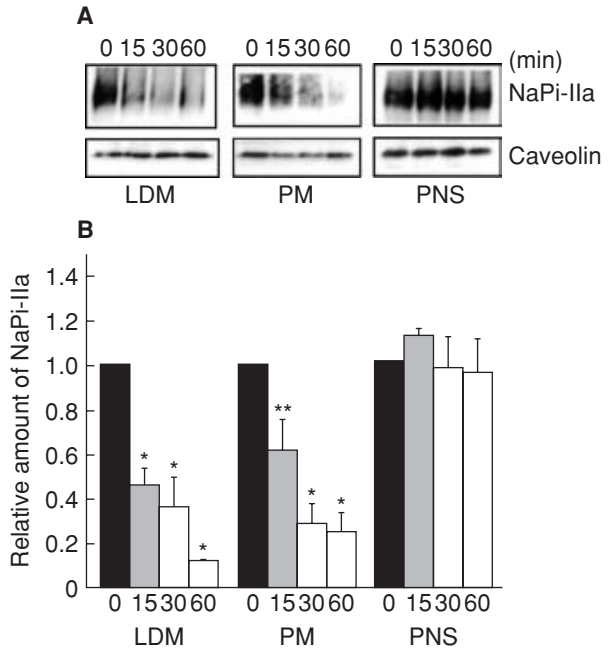


Fig. 3. Time-dependent effects of parathyroid hormone (PTH) on sodium-dependent phosphate transporter (NaPi-IIa) localization in the low-density membrane (LDM) domains. Opossum kidney (OK-N2) cells were incubated in the presence of 10^{-7} mol/L PTH for the indicated period of time. (*A*) Western blot analysis of time-dependent effects of PTH on localization of NaPi-IIa and caveolin in each cell fraction. PM is plasma membrane, PNS is postnuclear supernatant (time 0, 15, 30, and 60 minutes). The data presented are from three independent experiments. (*B*) Densitometric analysis of NaPi-IIa in (A). The data presented are from three independent experiments. All values are expressed as the mean ± SEM of triplicate smaples. The relative amount of NaPi-IIa at time 0 was 1. $^{*}P < 0.01$; $^{**}P < 0.05$ vs. time 0.

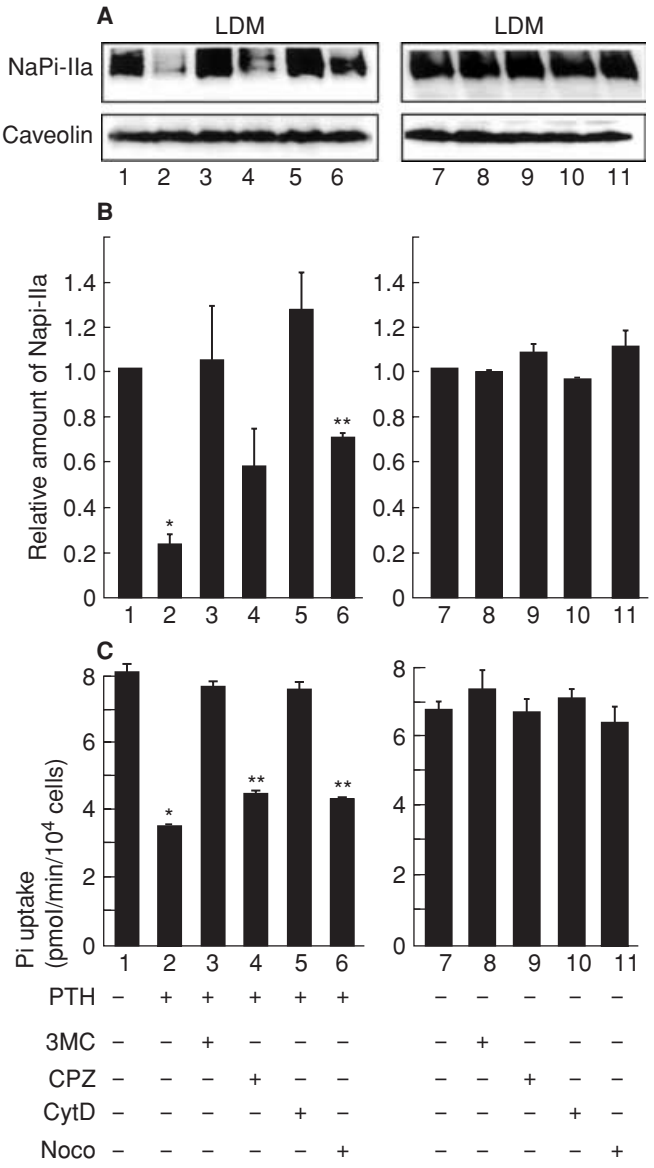


Fig. 4. Effects of 3-methyl-β-cyclodextrin, chlorpromazine, cytochalasin D, and nocodazole on the reduction of sodium-dependent phosphate transporter (NaPi-IIa) in the low-density membrane (LDM) domains (*A* and *B*) and on Pi uptake (*C*) in the presence and absence of parathyroid hormone (PTH). Opossum kidney (OK-N2) cells were preincubated with 0.5 mmol/L 3-methyl-β-cyclodextrin (3MC), 1 μmol/L chlorpromazine (CPZ), 0.5 μmol/L cytochalasin D (CytD), or 1 μmol/L nocodazole (Noco) for 5minutes, after which they were incubated with (lanes 2 to 6) or without (lane 1 and lanes 7 to11) PTH. (*A*) LDM domains were prepared and separated by gel electrophoresis (20 μg/lane) and were immunoblotted with anti-NaPi-II polyclonal antibody or anti-caveolin polyclonal antibody. (*B*) Densitometric analysis of the amount of NaPi-IIa. Values are the means ± SEM of triplicate samples. Relative NaPi-IIa amount at time 0 was 1. $^{*}P < 0.01$ vs. lane 1; $^{**}P < 0.05$ vs. lane 1 and $P < 0.01$ vs. lane 2. (*C*) Estimation of Pi uptake activity. Values are the means ± SEM of triplicate samples. $^{*}P < 0.01$ vs. lane 1; $^{**}P < 0.01$ vs. lane 1 and $P < 0.05$ vs. lane 2, respectively.

In order to determine whether ezrin was phosphorylated in response to PTH, we examined phosphorylation of its Thr567 residue. As shown in Fig. 8C, PTH reduced the amount of NaPi-IIa in the LDM domains (Fig. 8C, lane 2). PTH-dependent NaPi-IIa translocation was inhibited not only by 3-methyl-β-cyclodextrin (Fig. 8C, lane 3) but also Gö6976 (Fig. 8C, lane 4), a specific inhibitor of PKC, and H-89 (Fig. 8C, lane 5), a specific PKA inhibitor. Both Gö6976 and H-89 inhibited the phosphorylation of ezrin at Thr567 (Fig. 8C, lanes 4 and 5); this phosphorylation was not increased by PTH (Fig. 8C, lane 2). In consideration of the fact that PTH might enhance phosphorylation at sites other than serine/threonin residues, we immunoprecipitated ezrin from the LDM domains and then determined its degree of phosphorylation using antiphospho-(serine) PKC polyclonal antibodies and anti-phosho-(serine/threonine) PKA polyclonal antibodies. As shown in Figure 8D, both serine-phosphorylation by PKC and serine/threonine-phosphorylation by PKA was markedly increased after PTH treatment (Fig. 8D, lane 2); this phosphorylation was inhibited by specific inhibitors of PKC and PKA (Fig. 8D, lanes 4 and 5). On the other hand, 3-methyl-β-cyclodextrin did not affect the phosphorylation of ezrin (Fig. 8D, lane 3).

To investigate the role of ezrin in the PTH-mediated reduction of NaPi-IIa in the LDM domains, we transiently

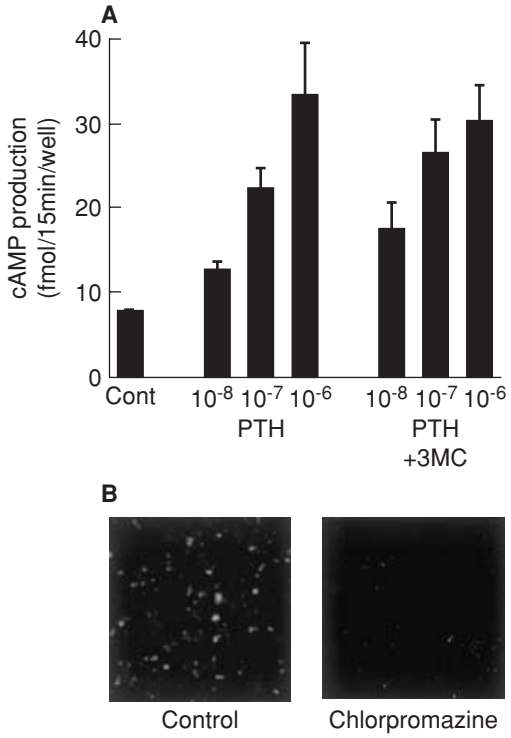


Fig. 5. Effects of 3-methyl-β-cyclodextrin on cyclic adenosine monophosphate (cAMP) production (*A*) and chlorpromazine on DiI-low-density lipoprotein (LDL) uptake (*B*) in response to parathyroid hormone (PTH) in opossum kidney (OK-N2) cells. (*A*) OK-N2 cells were treated with PTH (10^{-8} to 10^{-6} mol/L) in the presence or absence of 0.5 mmol/L of 3-methyl-β-cyclodextrin for 15 minutes, after which the amount of cAMP in these cells was determined. Values are expressed as the mean ± SEM of triplicate samples. (*B*) OK-N2 cells were incubated with or without 1 μmol/L of chloropromazine for 5 minutes before LDL uptake, after which they were incubated with 20 μg/mL DiI-LDL at 37°C for 30 minutes in the absence (control) or presence of 1 μmol/L chlorpromazine. The cells were fixed in 3% paraformaldehyde and processed for microscopy. Internalized DiI-LDL appeared as large vesicles scattered throughout the cell.

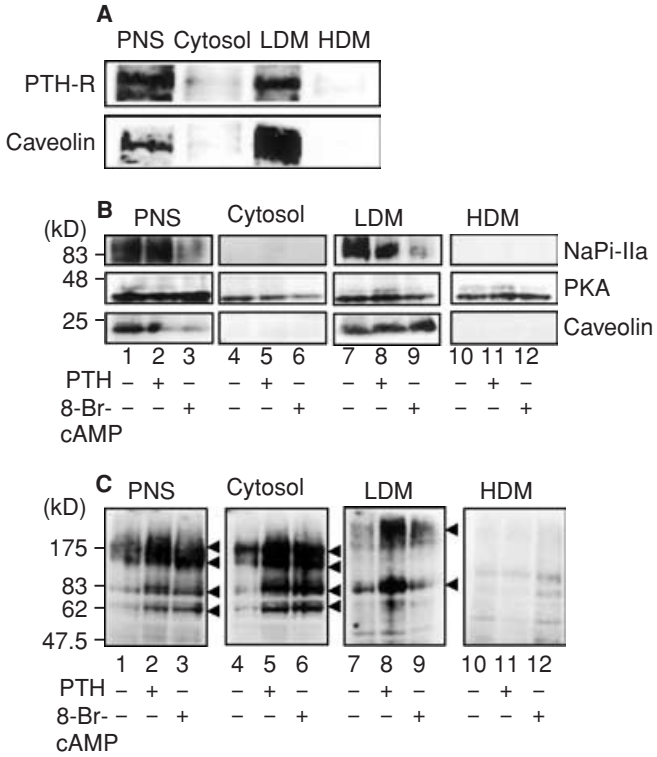


Fig. 6. Localization of parathyroid hormone (PTH) signaling molecules. (*A*) Submembrane localization of the PTH receptor. Opossum kidney (OK-N2 cells were partitioned in the indicated fractions and PTH receptor and caveolin were detected by western blotting. (*B* and *C*) Effects of PTH and 8-bromo-cyclic adenosine monophosphate (cAMP) on the subcellular localization of sodium-dependent phosphate transporter (pNaPi-IIa), protein kinase A (PKA), and caveolin (B) and on PKA substrate phosphorylation (C). OK-N2 cells were treated as follows: control (lanes 1, 4, and 7), 10^{-7} mol/L PTH (lanes 2, 5, and 8) for 15 minutes, or 200 nM 8-bromo-cAMP (lanes 3, 6, and 9) for 15 minutes. The bands indicated by the arrowheads are significantly greater than the control. The data presented are from three independent experiments. PNS is post nuclear supernatant.

expressed the N-terminal region of ezrin that was a dominant-negative ezrin, and found that such manipulation inhibited this PTH effect (Fig. 8E, lanes 3 and 4). Furthermore, we also found that dominant-negative ezrin reduced the amount of NaPi-IIa and endogenous ezrin (ezrin-WT) in the LDM domains (Fig. 8E, lane 1 vs. lane 3). Both NaPi-IIa and ezrin-WT expression level in the PNS had never changed by such manipulation (data not shown).

DISCUSSION

Our results suggest that NaPi-IIa as well as PTH-R, PKA, and PKC and their substrates are localized in the LDM domains of OK-N2 cells that corresponds to the location of lipid rafts and/or caveolae. The apical membrane of epithelial cells is enriched for cholesterol and sphingolipids, and this region also contains lipid rafts and caveolae [19]. In this study, caveolin which is a marker protein for caveolae was localized in the basolateral membrane of our renal proximal tubule cells, a finding that confirmed a previous report [20]; caveolin and most of the cell's NaPi-IIa was sublocalized in these cell's LDM domains. The fractioned LDM domains that we used contained membrane microdomains derived from both the apical and basolateral membrane, suggesting that the caveolin that we identified was likely to have been derived from the basolateral membrane. To exclude basolateral membrane contamination in our study, we investigated whether caveolin and NaPi-IIa were cofractionated in the LDM domains from apical membranes isolated from OK-N2 cells and the rat renal cortex, resulting that the cofractionation was observed (unpublished observations). Our data suggest that NaPi-IIa is localized in membrane microdomains that are biochemically similar to caveolae. Simons and Toomre [21] described caveolae as morphologically defined cell surface invaginations (containing caveolin) that

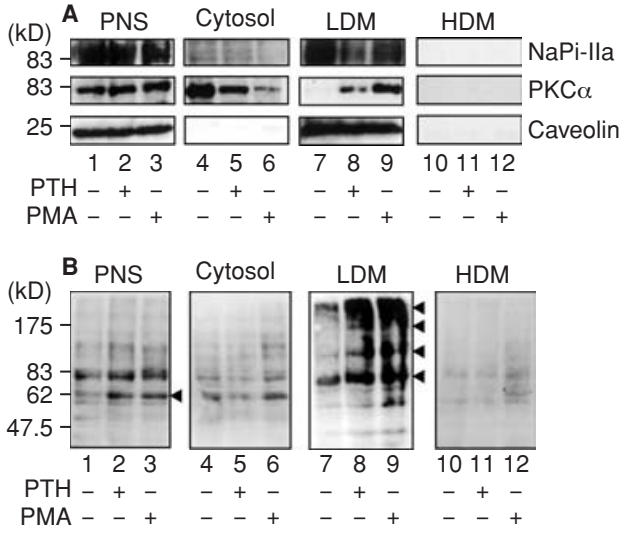


Fig. 7. Effects of parathyroid hormone (PTH) and phorbo 12-myristate 13-acetate (PMA) on the subcellular localization of sodium-dependent phosphate transporter (NaPi-IIa), protein kinase C (PKCα), and caveolin (*A*) as well as on PKC substrate phosphorylation (*B*). Opossum kidney (OK-N2) cells were treated with 10^{-7} mol/L PTH or 200 nmol/L PMA for 15 minutes. Lanes 1, 4, and 7 were control, lanes 2, 5, and 8 were 10^{-7} mol/L PTH, and lanes 3, 6, and 9 were 200 nmol/L PMA. The bands indicated by the arrowheads are significantly greater than the control. The data presented are from three independent experiments. PNS is post nuclear supernatant

were distinct from other membrane microdomains, even though these microdomains sorted into the low-density fractions due to their unique lipid composition. Our data suggest that LDM domains that contain NaPi-IIa should be classified as lipid rafts or some other membrane microdomains, rather than as caveolae. These observations are in agreement with the recent findings of Inoue et al [22] that NaPi-IIa partitioned in such a membrane microdomains in the rat kidney.

It is well known that, in response to PTH and other hormones, NaPi-IIa can be internalized by endocytosis and rapidly inhibit Pi reabsorption in renal proximal tubules [1–4]. We demonstrated that PTH decreased the levels of NaPi-IIa in the LDM domains of OK-N2 cells. We also found that 3-methyl-β-cyclodextrin blocked this effect and reduced PTH-induced Pi transport activity, though this compound did not inhibit PTH-dependent cAMP production or the phosphorylation of ezrin. Thus, methyl-β-cyclodextrin may block PTH-induced endocytosis but not the production of second messenger.

There are two possible mechanisms by which NaPi-IIa endocytosis occurs. The first is by clathrin-independent endocytosis that is mediated by the membrane microdomains. The second is by clathrin-dependent endocytosis. Traebert et al [23] demonstrated by immunoelectron microscopy that NaPi-IIa could be internalized by clathrin-coated pits. Our data, however, revealed only a nominal effect of chlorpromazine, an inhibitor of clathrin-coated pit formation on the down-regulation of NaPi-IIa by PTH. Although the relationship between clathrin-dependent endocytosis and clathrin-independent endocytosis vis-à-vis the down-regulation of NaPi-IIa is unclear, PTH-induced reductions in NaPi-IIa in the LDM domains could be the trigger of endocytosis in OK-N2 cells. Another possibility is that lateral trafficking of NaPi-IIa occurs between membrane microdomains and clathrin-coated pits, though we found no evidence of this.

Colocalization of NaPi-IIa in the LDM domains with the PTH receptor and other signaling molecules is probably important for the normal functioning of PTH, since many signaling molecules were reported to be compartmentalized in membrane microdomains; this compartmentalization was also found to be important for effective signal transduction [8, 10]. We confirmed that the signaling molecules PTH receptors, PKA, and PKC could localize in the same microdomain as NaPi-IIa and attempted to determine whether this microdomain also localized a specific phosphorylated substrate in response to PTH. Mineo et al [9] and Smart, Ying, and Anderson [24] showed that the compartmentalization of PKCα in caveolae was essential for their invagination; they also reported the localization of a 90 kD phosphorylatable molecule in these caveolae. In our study, we showed that both PKA and PKCα could be targeted to the LDM domains and be activated by PTH. We also identified two common substrates (80 and 250 kD) for both PKA and PKC in the LDM domains of OK-N2 cells, which have been implicated in NaPi-IIa inhibition.

We were able to identify the above, 80 kD molecule as ezrin. Ezrin is a member of ERM protein family, and is an 81 kD polypeptide that is rapidly phosphorylated at its tyrosine residues in response to epidermal growth factor [25]. It has also been shown that ezrin can be phosphorylated by both PKA [26] and PKCα [27]. Ezrin is generally localized in the cytosol, but phosphorylated ezrin localizes beneath the brush border membrane in epithelial cells and crosslinks the actin cytoskeleton to the plasma membrane [28]. In that capacity, it is thought to play a role in the regulation of cortical membrane structure [28], cell motility [27], and the internalization of receptor/transporters [29, 30]. Our data also showed that ezrin can localize to the LDM domains along with NaPi-IIa and that it is phosphorylated in response to PTH. Interestingly, the target moiety in ezrin that was phosphorylated in response to PTH was not threonine at 567, which is a well-characterized phosphorylation site in the molecule [31]. Recently, Zhao et al [32] showed that phosphorylation of ezrin at 567 was required for NHE3 recruitment to the apical membrane. The C-terminal of ezrin contains a binding site for F-actin (C-terminal 35 residues), suggesting that the phosphorylation of Thr567 was important for its interaction with F-actin [31]. Tsukita and

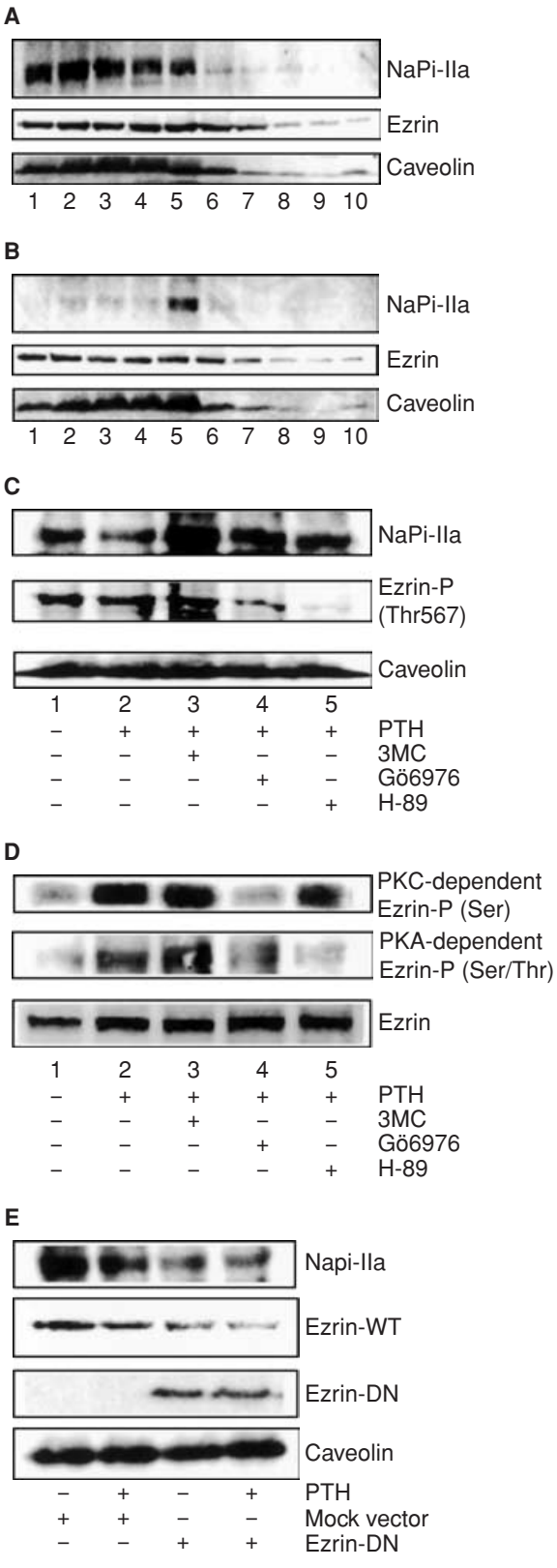


Fig. 8. Submembrane localization of ezrin and its role in the downregulation of sodium-dependent phosphate transport (NaPi-IIa) by parathyroid hormone (PTH). (*A* and *B*) Opossum kidney (OK-N2) cells were cultured in the presence or absence of 10^{-7} mol/L PTH for 20 minutes, after which they were subjected to OptiPrep gradient centrifugation analysis in a manner similar to that described in Figures 1A and B. (*C*) Effects of various inhibitors on PTH-dependent NaPi-IIa translocation and on the phosphorylation of ezrin at Thr567. OK-N2 cells were incubated in the absence (lane 1) or presence of 10^{-7} mol/L PTH (lanes 2 to 5) for 20 minutes as well as either 3-methyl-β-cyclodextrin (3MC) (lane 3), Gö6976 (lane 4), or H-89 (lane 5). Low-density membrane (LDM) domains were subjected to Western blot analysis using anti-NaPi-IIa polyclonal antibodies, antiphospho ezrin (Thr567) monoclonal antibodies, and anticaveolin polyclonal antibodies. (*D*) OK-N2 cells were treated as described (C), after which their LDM domains was prepared and immunoprecipitated using antiezrin monoclonal antibodies. The precipitants were subjected to Western blot analysis using antiphospho-serine protein kinase C (PKC) substrate polyclonal antibody, antiphospho-serine/threonine protein kinase A (PKA) substrate polyclonal antibody, and antiezrin monoclonal antibody. (*E*) Effect of dominant-negative ezrin (ezrin-DN) on the PTH-induced reduction in NaPi-IIa in the LDM fraction. Two days after either the mock or ezrin-DN expression vector was transiently transfected into OK-N2 cells, the cells were incubated in the presence or absence of 10^{-7} mol/L PTH for 30 minutes. The levels of NaPi-IIa, endogenous ezrin (ezrin-WT), ezrin-DN, and caveolin in the LDM domains were analyzed by Western blotting. The data presented are from three independent experiments.

Yonemura [33] also concluded that the C-terminal phosphorylation of ezrin helped to maintain its activated form. On the other hand, ezrin is known to have four potential PKA phosphorylation sites (Thr11, Ser249, Thr332, and Thr519) and seven PKC phosphorylation sites (Thr25, Thr98, Ser148 or 149, Thr358, Thr425, and Thr567), as confirmed using NetPhos 2.0. Our data suggest that PTH does not affect the phosphorylation of ezrin at Thr567, but increases its PKC-mediated phosphorylation at serine residues and PKA-mediated phosphorylation at serine/threonine residues. Specific inhibitors of PKA and PKC inhibited these phosphorylations as well as the PTH-induced reduction in NaPi-IIa. Thus, PTH may enhance phosphorylation of ezrin at the above sites rather than at Thr567, and these phosphorylations may be closely associated with the PTH-mediated reductions in NaPi-IIa.

Interestingly, NaPi-IIa was shown to indirectly interact with ezrin via NHERF-1 [also called ezrin binding protein-50 (EBP-50)] [34], supporting the notion that this protein may be an important intermediate in PTH action [35]. However, a recent report suggested that while NHERF-1 was important for the apical membrane targeting and expression of NaPi-IIa protein, endocytosis of NaPi-IIa in response to PTH/PKA/PKC did not require NHERF-1 [36]. NHERF-1 was shown to interact not only with transporters but also ion channels and G protein–coupled receptors, and to play a role in the formation of macromolecular complexes and signal transduction [37]. Mahon and Segre [38] reported that NHERF-1 interacted with the PTH receptor, phospholipase C, and actin in OK cells [38]. They also found that cytochalasin D disrupted the apical colocalization of both NHERF-1 and the PTH receptor, and inhibited PTH-mediated signaling [38]. In our study, cytochalasin D but not nocodazole inhibited

the PTH-induced reduction in NaPi-IIa, suggesting that cytoskeletal actin rather than microtubules were important for the rapid down-regulation of NaPi-IIa by PTH. Thus, ezrin may play an important role in the transmission of PTH-dependent cellular signals to actin that help to effect endocytosis.

To investigate the role played by ezrin in the down-regulation of NaPi-IIa by PTH, we transiently expressed the N-terminal half of ezrin (dominant-negative ezrin) in OK-N2 cells. This region contains the NHERF-1 binding site but not the actin binding site. Overexpression of this region of the molecule was reported to have a dominant-negative effect and to inhibit the morphogenesis of epithelial cells [39], bacterial entry into epithelial cells [40], and estrogen-regulated endocytosis of LDL [41]. Expression of dominant-negative ezrin resulted in the inhibition of the PTH-mediated reduction in NaPi-IIa in OK-N2 cells, suggesting that ezrin was necessary for the down-regulation of NaPi-IIa by PTH. Additionally, dominant-negative ezrin also interferred the targeting of NaPi-IIa into the LDM domains with inhibiting the targeting of endogenous ezrin, thus, ezrin would be important for targeting of NaPi-IIa to the membrane microdomains of plasma membrnane. Ezrin and NHERF-1 are likely to be important for the formation of the PTH receptor/NaPi-IIa/actin macromolecular complex in the microdomains in the apical membrane. Phosphorylated ezrin may modulate the NaPi-IIa/NHERF-1/actin complex, resulting in the eventual endocytosis of NaPi-IIa.

Membrane microdomains likely act as a platform for the macromolecular NaPi-IIa complex, signal transduction molecules (PTH receptor, PKA, and PKC), as well as cytoskeletal molecules. Ezrin is targeted to these microdomains and may play an important role in transforming PTH-dependent cellular signals into endocytosis.

ACKNOWLEDGMENTS

We thank Dr. Richard G.W. Anderson (University of Texas Southwestern Medical Center at Dallas, Texas) for his valuable discussions and comments. We also thank the technicians at the Support Center for Advanced Medical Sciences, University of Tokushima School of Medicine for their help with this project. This work was supported in part, by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology in Japan (to Y.T., H.Y., H.A., and E.T.), the Renal Osteodystrophy Research Foundation (to E.T.), and the 21st Century COE Program, Human Nutritional Science and Stress Control, Tokushima, Japan.

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